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The Role of Keratinocyte Differentiation in the Expression of Epitheliotropic Viruses

LORNE B. TAICHMAN, M.D., PH.D., SHEILA S. REILLY, AND ROBERT F. LAPORTA, PH.D.

Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York, U.S.A.

We have examined the growth of three epitheliotropic viruses in cultures of human epidermal keratinocytes: herpes simplex virus (HSV) type 1, adenovirus type 2 (Ad-2), and human papillomavirus (HPV) type 1. Differences were noted in the level of expression of each virus, and these differences may be related to a dependency or lack of dependency on keratinocyte differentiation for complete viral growth.

Of the three viruses studied, HSV was the only one to replicate productively in all cells of the culture. Its expression was independent of keratinocyte differentiation. This is unlike Ad-2, which infected all cells in the culture but replicated productively only in the suprabasal cells. Basal keratinocytes were shown to be infected, but for unknown reasons, they appeared in most instances to be nonpermissive for Ad-2 replication. Infected basal keratinocytes became permissive when they reached a suprabasal position. Ad-2 appears to require keratinocyte differentiation for full expression in culture.

Following infection with HPV, cultured keratinocytes showed no evidence of productive replication. However, 50 to 250 copies of HPV DNA could be detected in each cell (average) as stable nonintegrated molecules. Viral DNA replication has been shown to occur in the younger cells and not in the older, more differentiated keratinocytes. The failure of HPV to be fully expressed in culture may be related, in part, to incomplete differentiation of the keratinocyte in vitro.

The major conclusions of this study are (1) that keratinocyte differentiation is likely to play a role in the expression of some epitheliotropic viruses in culture, and (2) that keratinocyte differentiation may be a factor in the pathogenesis of certain viral diseases of keratinizing epithelia.

In this article we wish to draw attention to a relatively new approach to the study of keratinocyte biology that uses epitheliotropic viruses. Over the past few decades, viruses have proven extremely useful in the investigation of gene regulation in eukaryotic cells [1]. Their usefulness stems from two facts: first, viruses and their associated products can be detected with relative ease against a background of cellular molecules, and second, since viruses are dependent on the metabolic machinery of the host cell, expression of viral genes reflects the nature of host control mechanisms. However, the lack of methods for culturing cells other than fibroblasts and aneuploid lines has meant that studies with specific cell types, such as the keratinocyte, have not been possible. The recent development of methods for routine cultivation of keratinocytes now permits application of the knowledge and techniques of virology to the study of keratinocyte biology.

Our specific goal has been to elucidate factors that control differentiation of keratinocytes. The basic approach has been to look for and examine the replication of viral agents whose expression in cultured epidermal keratinocytes appears to be blocked or influenced by the state of cellular differentiation. In this article we summarize past work and report recent findings on the expression of three epitheliotropic viruses: adenovirus type 2 (Ad-2), human papillomavirus (HPV) type 1, and herpes simplex virus (HSV) type 1. The expression of each virus is different, and in the case of adenovirus and human papillomavirus, their expression appears to require differentiation of the keratinocyte.

MATERIALS AND METHODS

Human foreskin keratinocytes were cultured by the method of Rheinwald and Green [2] with some modifications [3,4]. Sources of adenovirus type 2 (Ad-2) and human papillomavirus (HPV) type 1 and methods for infection have been described elsewhere [5,6]. Herpes simplex virus (HSV) type 1 was a gift from Dr. Bill Kilpatrick (Wake Forest University) and was passaged in WI-38 cells.

Immunofluorescence microscopy was performed using antibody to Ad-2 [5] and antibody to HPV [6]. Antibody to HPV was produced in rabbits after purifying HPV particles by two cycles of centrifugation in CsCl density gradients and one cycle in a sucrose density gradient. The IgG fraction of immune serum was isolated and used at a dilution of 1:250. Fractionation of keratinocytes in gradients of Ficoll 400 has been described [4].

Techniques for DNA isolation, Southern blotting, and hybridization with ^{32}P-labeled HPV DNA have been detailed elsewhere [6]. RNA was isolated from keratinocytes 4 days after infection and 4 hours after the addition of 10 μM anisomycin [7].

RESULTS AND DISCUSSION

Adenovirus

Adenoviruses are DNA viruses associated with infections of respiratory epithelia, intestinal tract, and conjunctiva. Adenovirus type 2 (Ad-2) can be isolated from cultures of adenoid tissue and are associated with manulopapular rashes in the skin of infants. The organization of the Ad-2 genome and its expression have been studied in considerable detail [8]. Basically, Ad-2 is a virus whose genes are expressed in a regulated temporal sequence and whose RNA transcripts are subject to rearrangement by splicing mechanisms.

Ad-2 replication is blocked in basal keratinocytes but not in suprabasal cells. Two to 3 days following infection of mature cultures of epidermal keratinocytes with Ad-2, cells become retracted and rounded up (Fig. 1*B*). Electron microscopy and indirect immunofluorescence show that these cells contain intranuclear viral particles, viral capsid antigens, and 54 K antigen [5]. (The 54 K antigen is a viral protein synthesized early in the course of infection in permissive cells.) However, a striking observation is the presence of an underlying stratum of basal keratinocytes that appear normal morphologically (Fig. 1*B*) and possess no detectable 54 K or capsid antigen. These normal-appearing cells persist and replicate, and when proliferation progresses to the point where stratification occurs, the resulting

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Reprint requests to: Ms. Sheila S. Reilly, Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794.

Abbreviations:
EDTA: ethylenediaminetetraacetic acid
Ad-2: adenovirus type 2
HPV: human papillomavirus
HSV: herpes simplex virus

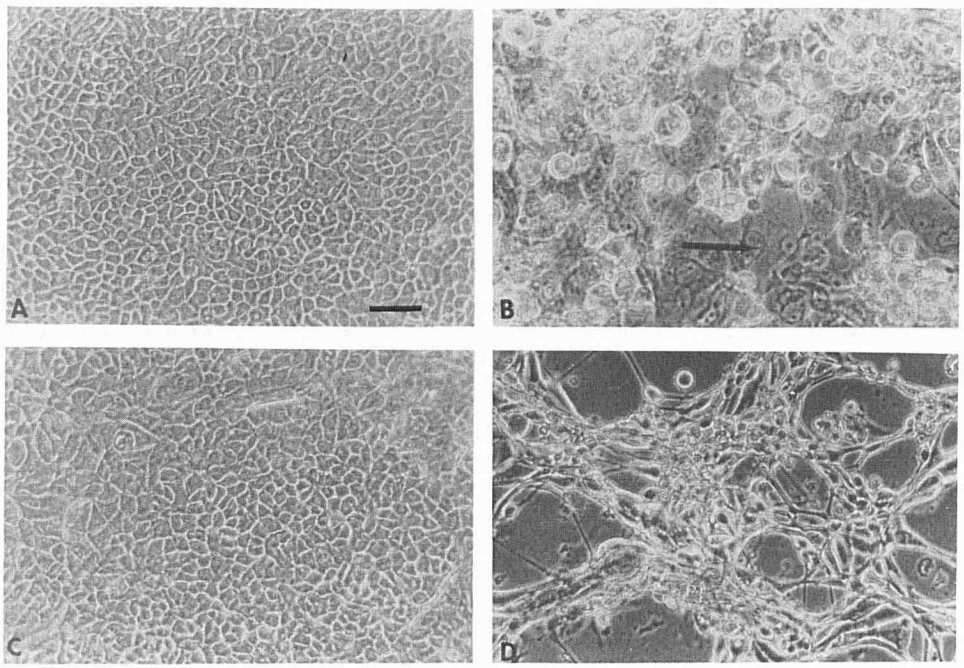


FIG 1. Appearance of keratinocytes infected with epitheliotropic viruses. *A*, Uninfected keratinocytes; bar = 100 μm. *B*, Keratinocytes 21 days after infection with Ad-2. Note the underlying stratum of normal-appearing basal cells (*arrow*). *C*, Keratinocytes 28 days after infection with HPV. *D*, Keratinocytes 54 hours after infection with HSV.

suprabasal cells develop the cytopathic effect described above.

Experiments have shown that the lack of response of basal keratinocytes to Ad-2 cannot be explained by insufficient virus in the medium nor by limited access to basal cells [5]. Basal keratinocytes can be infected with Ad-2 but appear to be nonpermissive for viral replication. To demonstrate conclusively that basal keratinocytes harbor the virus in a repressed state, keratinocytes were infected while in suspension, washed, and seeded into culture dishes in the presence of Ad-2 neutralizing antibody. Neutralizing antibody inactivates any free viral particles in the medium. Twelve hours later, cultures were rinsed with PBS and cultivation continued in the presence of neutralizing serum. Adherent cells proliferated and formed small colonies that appeared normal (Fig. 2*A*). However, when these cells differentiated and became suprabasal, they developed a cytopathic response and were positive for viral capsid antigen (Fig. 2*B*, *C*). The only way for suprabasal cells to have acquired the virus was to have harbored it at the outset, as basal keratinocytes. Therefore, Ad-2 must have infected basal cells, but it must have been prevented from replicating in these cells. The mechanism of repression in basal cells is unknown; however, the event that releases the block to Ad-2 replication may be a step involved in keratinocyte differentiation. Exposure of infected basal keratinocytes to 0.02% EDTA for 10 to 15 minutes induced productive replication of Ad-2 in basal keratinocytes. The significance of this observation is unclear.

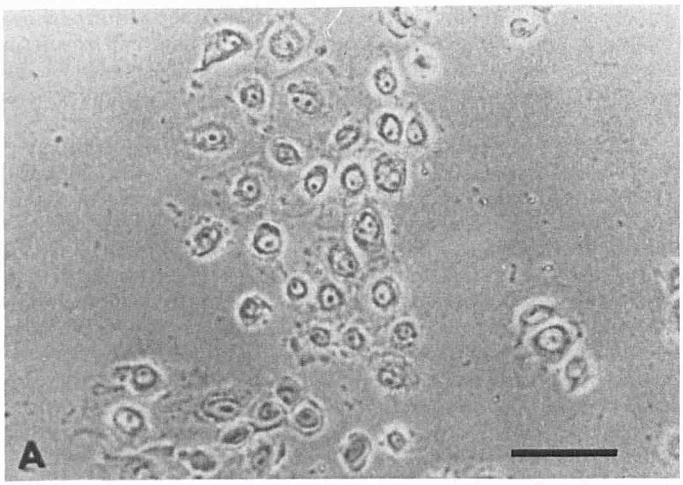
A small number of basal keratinocytes do have Ad-2 particles. Careful examination of thin sections of infected cultures reveals the presence of viral particles in the nuclei of a small number of basally located cells [5]. Since Ad-2 replicates productively in the more differentiated suprabasal cells, the presence of Ad-2 in some basal cells may signify that these cells have entered the terminal differentiation program but have not as yet left the basal layer. Watt and Green [9] have observed that a small number of basal keratinocytes contain involucrin when terminal differentiation is blocked by the use of low-calcium medium. These findings suggest, but do not prove, that terminal differentiation begins in basal keratinocytes. The question of when terminal differentiation begins remains an important, yet unsolved, problem in keratinocyte biology.

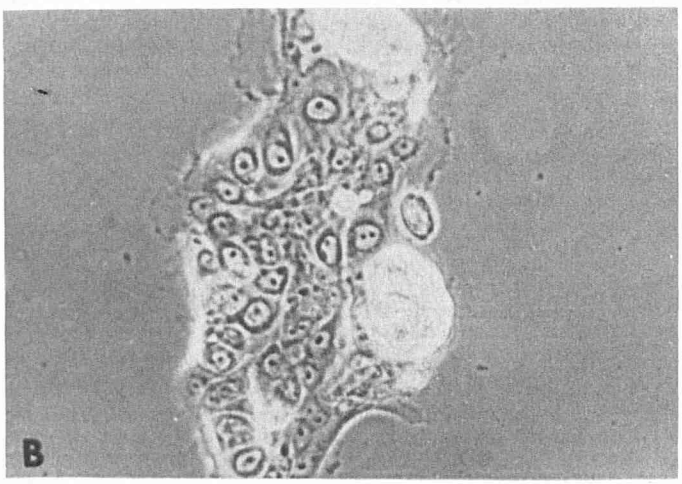
Malignant keratinocytes are permissive for Ad-2. Rheinwald and Beckett [10] have described the properties of a number of lines of malignant keratinocytes derived from human squamous cell carcinomas. One of the characteristics of these cells is their inability to stratify. Since normal basal keratinocytes are nonpermissive for Ad-2, it is possible to test the basal-like character of malignant keratinocytes by their response to Ad-2. Monolayers of two lines of malignant keratinocytes (SCC nos. 9 and 25 from Dr. James Rheinwald) were infected with Ad-2, and within 2 to 3 days, a cytopathic effect was clearly evident in all cells. Therefore, these malignant cells are not simply basal keratinocytes, but must be blocked at some point in the differentiation pathway that allows Ad-2 replication but not stratification. Whatever cellular event is responsible for the acquisition of a permissive phenotype, this step occurs prior to stratification. The use of a virus to define "normal" basal keratinocytes may provide a novel means of mapping the early steps in keratinocyte differentiation.

Human Papillomavirus (HPV)

HPV constitutes a group of DNA viruses known to induce benign proliferations of keratinocytes resulting in warts and condyloma [11, 12]. Malignant conversions are rare and occur in unusual conditions, such as epidermodysplasia verruciformis [13]. Productive replication of this virus wart tissue is thought to require specific differentiation of the keratinocyte [12] because viral antigens cannot be detected until the upper portions of the stratum spinosum, whereas viral DNA can be detected as deep as the first suprabasal layer. In addition, HPV particles can be recovered in abundance from lesions on surfaces that are normally highly keratinized (such as plantar and palmar skin), but not from lesions on less keratinized surfaces (such as genital and laryngeal epithelia).

HPV persists as a stable plasmid in cultured keratinocytes. When cultures of epidermal keratinocytes are infected with HPV particles, there is no evidence of productive infection, i.e., no cytopathic response (Fig. 1*C*), no detectable viral antigens, and no recoverable viral particles even after prolonged cultivation [6]. When total cellular DNA is extracted from these cells, cut with restriction endonucleases, and analyzed by Southern hybridization, HPV DNA is clearly detected up to eight passages after infection (Fig. 3*A*). Examination of endonuclease fragments of viral DNA in Fig. 3*A* reveals that the bands of viral DNA from infected cells migrate in the same position as DNA from intact virions. Had viral DNA in the cell been integrated into the host chromosome, the electrophoretic mobility of some viral bands would have been altered by





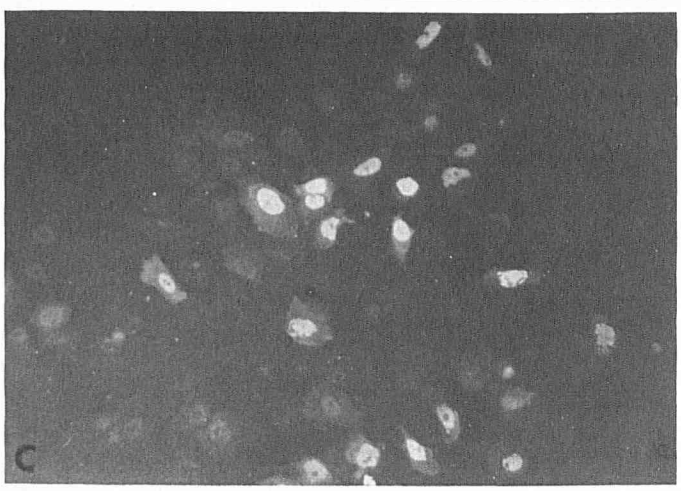
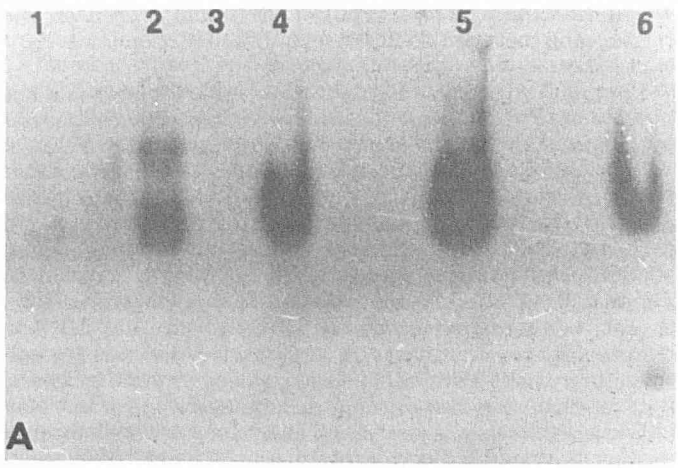


FIG 2. Growth of keratinocytes following infection with Ad-2. Keratinocytes were infected with Ad-2 while the cells were in suspension, rinsed, and cultured in the presence of Ad-2 neutralizing antibodies. *A*, Small colonies are evident 3 days after infection; bar = 100 μm. *B*, By 7 days after infection, suprabasal cells arise and these cells develop a cytopathic effect. *C*, When stained with Ad-2 FITC antibody, they show a positive fluorescence. Suprabasal cells fluoresce positively.

attached sequences of cellular DNA. Therefore, within the limits of detection by Southern hybridization, all HPV DNA exists in keratinocytes as nonintegrated episomes. By comparing the intensity of viral bands to bands produced by mixtures of known amounts of viral and cellular DNA, it is possible to estimate the average number of copies of HPV to be between 50 and 250 per cell. We do not know if all cells in the culture are infected or if all infected cells contain the same number of copies of HPV DNA. To determine the physical form of viral DNA in the cell, Southern blotting was repeated using Sal 1, an endonuclease that does not cleave HPV DNA. The results (Fig. 3*B*) show that most of the viral DNA is present as supercoiled and nicked circular molecules with a small fraction of linear molecules.

HPV DNA replication is probably linked to cellular replication. Replication of HPV DNA in infected keratinocytes has been demonstrated directly by the incorporation of [^{3}H]thymidine into viral-specific DNA [6]. Recent experiments [7] involving fractionation of keratinocytes based on their maturational age have also shown that the number of copies of HPV DNA per cell does not increase as keratinocytes undergo maturation in culture. This means that viral DNA replication takes place only in the youngest cells. Had viral DNA replication continued throughout keratinocyte maturation, HPV DNA would have accumulated and been present in increased amounts in the older cells. Replication of keratinocyte DNA occurs only



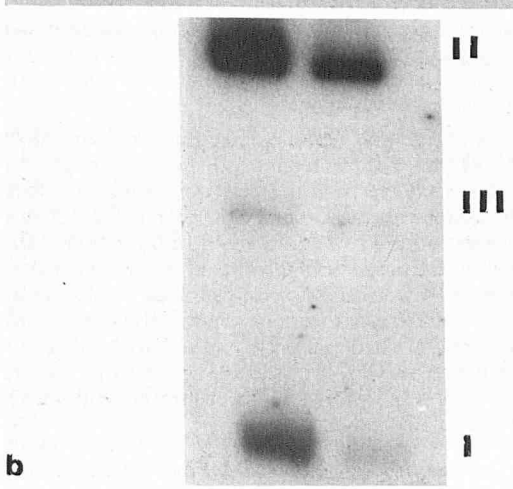


FIG 3. HPV DNA in cultured keratinocytes. *A*, HPV DNA persists in replicating keratinocytes. Total cellular DNA was extracted from keratinocytes one, three, and eight passages after infection. The DNA was then treated with Bam H1 endonuclease and analyzed by Southern hybridization. Lane 1 is a positive control containing DNA from HPV particles. Lane 2 is a negative control containing DNA from uninfected cells. Lanes 3, 4, and 5 contain DNA from cells one, three, and eight passages after infection, respectively. (Reproduced by permission of authors from [6].) *b*, Structure of HPV DNA in infected keratinocytes. DNA from infected keratinocytes was treated with Sal 1 endonuclease (which does not cleave HPV-1 DNA) and analyzed by Southern hybridization. The three bands correspond to supercoiled circular DNA (form I), linear DNA (form III), and circular DNA nicked in one strand (form II). The left lane contains twice the amount of DNA as the right one.

in the youngest cells of the culture [4,14]. Therefore, HPV DNA replication is probably linked to cellular replication.

Enhancing the level of keratinocyte differentiation does not lead to productive HPV infection. The failure of HPV to be fully expressed in cultured keratinocytes may be related to the incomplete level of keratinization achieved by these cells in vitro. To test this hypothesis, infected cells were grown in two ways known to enhance keratinocyte differentiation in vitro. First, infected cells were cultured with delipidized serum [15], and second, infected cells were injected into the subcutaneous tissue of athymic mice [16]. In neither case was there morphologic evidence to suggest the presence of a cytopathic effect or viral particles [17]. The validity of these results is questionable, since, in the first instance, the uninfected keratinocytes grown in delipidized serum did not possess well-defined keratohyalin granules or the 67 K keratin protein as reported by Fuchs and Green [15]. In the second instance, the cysts produced in athymic mice were harvested 3 weeks after injection. Complete HPV expression may require a longer period, but resorption of the cysts makes prolongation of the experiment impossible. Failure to achieve productive replication of HPV in these experiments does not provide definitive information on the relationship between keratinocyte differentiation and HPV expression.

In exploring this relationship further, other factors should be considered. First, it is unclear what percentage of keratinocytes are actually infected by HPV. If only a very small fraction of cells are infected, then a positive result may not be detectable. Second, virus production in wart tissue occurs in transformed keratinocytes, not in normal keratinocytes, which were used in this study. Productive replication of HPV in vitro may require an additional event, such as benign transformation. Third, different types of papillomaviruses have been recovered from specific lesions and specific sites [13]. Growth of an HPV in culture may require utilization of keratinocytes from the epithelium normally affected. Since keratinocytes are the natural host for HPV, the lack of complete expression in culture may indicate that some aspect(s) of keratinocyte metabolism is missing or altered in the culture situation. The search for ways to augment the expression of HPV may lead to an enhanced understanding of keratinocyte differentiation.

Herpes Simplex Virus (HSV) Type 1

HSV is a DNA virus that induces papular and vesicular lesions primarily of the oral mucosa and epithelium at the mucocutaneous border [18]. When HSV was used to infect cultures of epidermal keratinocytes, all the cells in the culture became swollen and rounded within 36 to 48 hours (Fig. 1*D*). These cells became retracted and produced a cobweb cytopathic appearance. In thin sections, viral particles were seen in nuclei, adjacent to the nuclear envelope, and in the cytoplasm [17]. In the cytoplasm, viral particles were surrounded by portions of the nuclear membrane. The replication of HSV in cultured keratinocytes is complete and is not limited in any way by the state of cellular differentiation.

Significance of Differentiation-Dependent Viral Expression

The major conclusion of these studies is that keratinocyte differentiation is likely to play a role in the expression of some epitheliotropic viruses. The concept that the state of differentiation of a cell influences the expression of an invading virus is a relatively new one and may help to explain certain aspects of viral disorders of keratinizing epithelia, such as persistent viral infections or viral latency. For example, if lytic viral replication occurs only in differentiated cells, then the undifferentiated stem cell population could escape injury and could continue to serve as a reservoir of infected cells. In this way, persistent viral infections might arise. In another example, if viral replication is dependent on certain aspects of cell differentiation, then the absence of these key cellular events could result in limited viral expression and possibly latency. Conversely, acquisition of these differentiated traits in latently infected tissue could result in changeover to productive viral replication. These hypotheses merit examination in the pathogenesis of certain viral infections of keratinizing epithelia, especially HPV infections.

Since viruses depend on the metabolic machinery of the host cell, the more genetic information encoded in the viral genome, the less this dependency need be. In the three viruses examined, there was a gradation of dependency: HSV was fully expressed in all keratinocytes, Ad-2 achieved full expression only in suprabasal or more differentiated cells, and HPV achieved only a limited expression in all the cells in the culture. The genome size for these three viruses is 90–120, 36, and 7.8 kb, respectively, and therefore, their coding capacity differs accordingly. It is possible that the smaller genomes of Ad-2 and HPV make these viruses more dependent than HSV on host enzymes. In the case of infected keratinocytes, reactions needed for viral replication may be the same as those involved in differentiation of the cell. This would mean that viral agents could be used to probe the process of keratinocyte differentiation. With the wealth of manipulative techniques available to the virologist, such an approach might be a very fruitful one.

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